



## Successful transfer of chemical-induced asthma by adoptive transfer of low amounts of lymphocytes in a mouse model

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### ABSTRACT

**Background:** We optimized an adoptive transfer protocol in our mouse model of TDI-induced asthma in order to investigate the mechanisms of this type of occupational asthma.

**Methods:** On days 1 and 8, BALB/c mice were dermally sensitized with 0.3% TDI or vehicle (acetone/olive oil), and on day 15, they were sacrificed and a cell suspension was made from auricular lymph nodes. First,  $0.1 \times 10^6$ ,  $0.5 \times 10^6$ ,  $1 \times 10^6$  or  $5 \times 10^6$  cells were injected intravenously into naïve mice and three days later these mice received an oropharyngeal challenge with 0.01% TDI or vehicle. Second, mice were challenged with 0.01% TDI 1, 3, 5 or 7 days after transferring  $0.5 \times 10^6$  cells. The following endpoints were measured one day after challenge: methacholine reactivity; differential cell counts in bronchoalveolar lavage (BAL) and total serum IgE.

**Results:** Naïve mice receiving  $0.5 \times 10^6$ ,  $1 \times 10^6$  or  $5 \times 10^6$  cells showed significant increases in airway reactivity one day after TDI challenge; BAL neutrophils were increased after transferring  $0.5 \times 10^6$  and  $1 \times 10^6$  cells. A TDI challenge 3 days after transferring  $0.5 \times 10^6$  cells gave a 3-fold increase in airway resistance and a pronounced airway inflammation, whereas challenging at other time points gave no differences.

**Conclusion:** We were able to passively sensitize naïve mice using lymph node cells from TDI-sensitized mice, resulting in an asthma-like response after an airway challenge. In comparison to other adoptive transfer protocols we used substantially lower number of cells to obtain the desired response.

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### 1. Introduction

The prevalence rates of allergic conditions, including asthma, have increased significantly throughout the world in the past 50 years (Traidl-Hoffmann et al., 2009). It is generally accepted that 9–15% of adult asthma can be linked to workplace exposures, and recent data indicate that 25% or more of de novo asthma in adults may have an occupational basis (Dykewicz, 2009). Both high molecular weight and low molecular weight agents (LMW) are defined as possible asthmogens. At present, there are approximately 100,000 chemicals and new LMW chemicals are frequently introduced into the market. Some LMW agents induce occupational asthma through

IgE-mediated mechanisms, but others may lead to airway sensitization through non-IgE-mediated immunologic mechanisms that are not completely understood (Dykewicz, 2009; Malo and Chan-Yeung, 2009).

Isocyanates are one of the most common causes of occupational asthma. A large variety of isocyanates and their prepolymers are widely applied in paints and varnishes, elastomers, and the manufacture of flexible and rigid foams and fibers (Dykewicz, 2009; Malo and Chan-Yeung, 2009).

We have developed and validated a mouse model of chemical-induced asthma, in which we dermally sensitize the mice to toluene diisocyanate (TDI) and later give them a single intranasal or oropharyngeal challenge with TDI (De Vooght et al., 2009; Tarkowski et al., 2007; Vanoirbeek et al., 2004, 2006, 2008, 2009). This study is part of our efforts to understand the mechanisms of this particular form of asthma.

In this experimental setup we evaluated whether it would be possible to transfer the sensitization to TDI using cells. We explored different experimental setups to establish (1) the optimal amount of auricular lymph node cells to be transferred and (2)

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the optimal time point to challenge the mice after transferring the cells.

We were able to passively sensitize naïve mice with lymph node cells from TDI-sensitized mice and this yielded an asthma-like response after an airway challenge. A novelty of our findings is that we achieved this with a much lower amount of cells than is described in the literature.

## 2. Materials and methods

### 2.1. Reagents

Toluene-2,4-diisocyanate (98%; Fluka, CAS 584-84-9), acetyl- $\beta$ -methylcholine (methacholine) and acetone were obtained from Sigma–Aldrich (Bornem, Belgium). Pentobarbital (Nembutal®) was obtained from Sanofi Santé Animale (CEVA, Brussels, Belgium) and Isoflurane (Forene®) from Abbott Laboratories (S.A. Abbott N.V., Ottignies, Belgium). The vehicle (acetone/olive oil, AOO) used to dissolve TDI consisted of a mixture of 2 volumes of acetone and 3 volumes of olive oil (Selection de Almazara, Carbonell, Madrid, Spain) for the dermal sensitization, and 1 volume of acetone and 4 volumes of olive oil for the oropharyngeal challenge. Concentrations of TDI are given as percent (v/v) in AOO.

### 2.2. Animals

Male BALB/c mice (approximately 20 g, 6 weeks old) were obtained from Harlan (Horst, The Netherlands). The mice were housed in a conventional animal house with 12-h dark/light cycles. They were housed in filter top cages and received lightly acidified water and pelleted food (Trouw Nutrition, Gent, Belgium) ad libitum. All experimental procedures were approved by the Local Ethical Committee for Animal Experiments.

### 2.3. Experimental setup

On days 1 and 8, the animals received dermal applications with 0.3% TDI or with the vehicle on the dorsum of both ears (20  $\mu$ l/ear). On day 15, these mice were sacrificed, blood was taken and auricular lymph nodes were pooled and processed to obtain a cell suspension. Cell suspensions were obtained by pressing the lymph nodes through a cell strainer (100  $\mu$ m) (BD Bioscience, Erembodegem, Belgium) and rinsing with 10 ml tissue culture medium (RPMI-1640) (Invitrogen, Merelbeke, Belgium). Cells were counted using a Bürker hemocytometer. Lymphocytes were washed three times and suspended in HBSS<sup>+</sup> buffer.

#### 2.3.1. Characterizing the cell suspension

**2.3.1.1. Flow cytometry.** Five-hundred thousand cells were stained with anti-CD3 (APC), anti-CD4 (APC-Cy7), anti-CD8 (PerCP-Cy5.5) and anti-CD25 (PE) or received a single staining with anti-CD19 (PE) or a double staining with anti-CD19 (PE) and anti-MHCII (APC, Miltenyi Biotec, Utrecht, The Netherlands) labelled antibodies, according to standard procedures, and with control samples being labelled with isotype match control antibodies (BD Biosciences, Erembodegem, Belgium). Flow cytometry (Facsarray, BD Biosciences, Erembodegem, Belgium) was performed using at least  $10^5$  cells.

#### 2.3.2. Optimizing the amount of cells to be transferred

$0.1 \times 10^6$ ,  $0.5 \times 10^6$ ,  $1 \times 10^6$  or  $5 \times 10^6$  cells were injected, in a total volume of 250  $\mu$ l, intravenously in the tail vein of naïve mice. Three days after the transfer of lymph node cells to these mice, they received an oropharyngeal challenge with

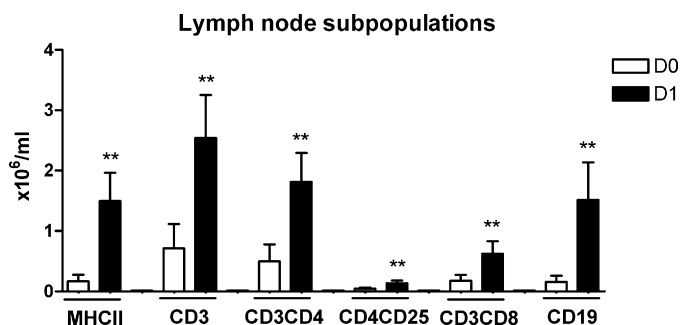


**Fig. 1.** Characterizing the cell suspension. Freshly isolated lymphocytes in the auricular lymph nodes were analysed by flow cytometry using the following markers: anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-CD19 and anti-MHCII. Data are presented as means  $\pm$  SD,  $n=4-7$  per group. \*\* $p<0.01$  compared with the D0 group. Experimental groups are D0 and D1, where D0 are lymph node cells of donor mice (D) dermally treated with AOO, and D1 are lymph node cells of donor mice dermally treated with TDI.

0.01% TDI or vehicle. Each treatment group consisted of 5–6 animals. Mice were sacrificed 24 h after the challenge.

Experimental groups: D0R0  $0.1 \times 10^6$ , D1R1  $0.1 \times 10^6$ , D0R0  $0.5 \times 10^6$ , D1R1  $0.5 \times 10^6$ , D0R0  $1 \times 10^6$ , D1R1  $1 \times 10^6$ , D0R0  $5 \times 10^6$  and D1R1  $5 \times 10^6$ . D0 and D1 represent the lymphocytes from donor (D) animals that received on days 1 and 8 the vehicle (D0) or TDI (D1). These lymphocytes were then transferred into naïve recipient (R) mice which received a challenge with vehicle (R0) or TDI (R1) three days after the transfer. Briefly, group D0R0 are lymph node cells of mice dermally treated with AOO injected in naïve mice, which received an AOO challenge and group D1R1 are lymph node cells of mice dermally treated with TDI injected in naïve mice, which received a TDI challenge.

#### 2.3.3. Optimizing the time point for challenge after the transfer

With the results found in the first part of the experiments, we decided to proceed with transferring  $0.5 \times 10^6$  lymph node cells to naïve mice. One (d1), three

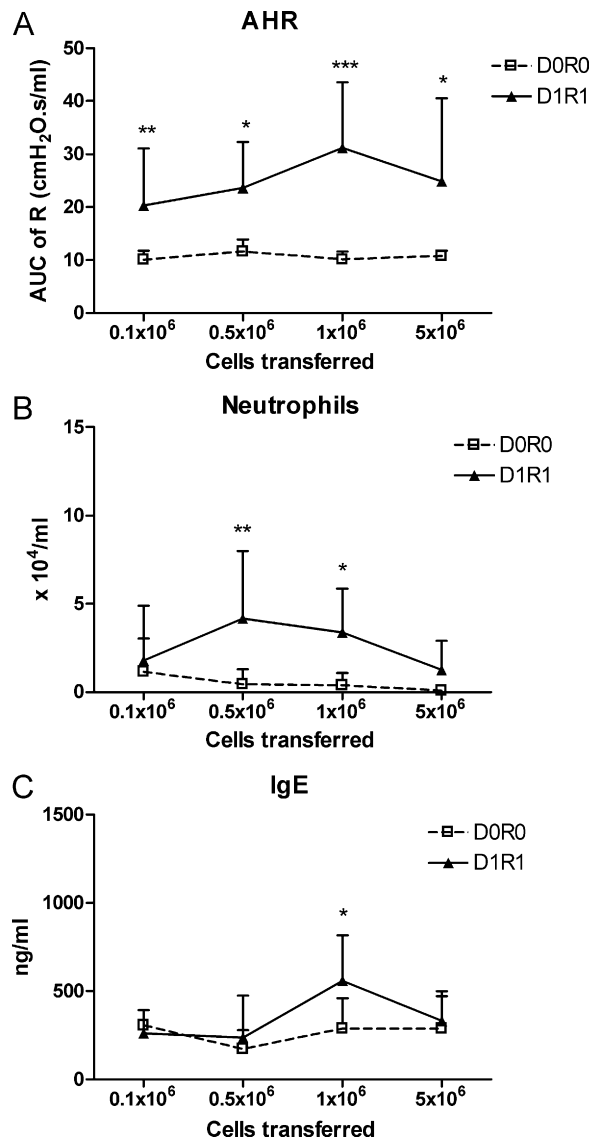


**Fig. 2.** Optimizing the amount of cells to be transferred. The airway resistance (R), after increasing concentrations of methacholine (0–10 mg/ml), was measured 24 h after the challenge. The AUC of R was calculated (A). B gives the total numbers of neutrophils in the BAL fluid 24 h after challenging with the vehicle or TDI. Furthermore, blood was collected 24 h after the challenge and total serum IgE was measured using a standard ELISA (C). Data are presented as means  $\pm$  SD,  $n=5-6$  per group. \* $p<0.05$ , \*\* $p<0.01$  and \*\*\* $p<0.001$  compared with their own control group. Experimental groups are D0R0  $0.1 \times 10^6$ , D1R1  $0.1 \times 10^6$ , D0R0  $0.5 \times 10^6$ , D1R1  $0.5 \times 10^6$ , D0R0  $1 \times 10^6$ , D1R1  $1 \times 10^6$ , D0R0  $5 \times 10^6$  and D1R1  $5 \times 10^6$ . Group D0R0 are lymph node cells of donor mice dermally treated (days 1 and 8) with AOO injected in recipient (R) naïve mice, which received an AOO challenge and group D1R1 are lymph node cells of donor mice dermally treated with TDI injected in recipient naïve mice, which received a TDI challenge.

(d3), five (d5) or seven (d7) days after the transfer they received an oropharyngeal challenge with 0.01% TDI or vehicle. Each treatment group consisted of 5–6 animals. Experimental groups are D0R0 d(day)3, D1R1 d1, D1R1 d3, D1R1 d5 and D1R1 d7. The abbreviations are the same as in the first experimental setup.

## 2.4. Ventilatory function

### 2.4.1. Airway reactivity to methacholine

Twenty-four hours after the challenge, reactivity to methacholine was assessed invasively using a forced oscillation technique (FlexiVent, SCIREQ, Montreal, Canada) (Vanoirbeek et al., 2010). As previously described, airway resistance ( $R$ ) was measured using a “snapshot” protocol. For each mouse,  $R$  was plotted against methacholine concentration (0–10 mg/ml) and the area under the curve (AUC) was calculated (Vanoirbeek et al., 2010).

### 2.5. Pulmonary inflammation (bronchoalveolar lavage)

After the measurements of airway reactivity to methacholine, the mice were deeply anesthetized by an intraperitoneal injection of pentobarbital (90 mg/kg body weight). Blood was first taken from the retro-orbital plexus, centrifuged ( $14,000 \times g$ , 10 min) and serum samples were stored for further analyses. The lungs were lavaged, in situ, three times with 0.7 ml sterile saline (0.9% NaCl), and the recovered fluid was pooled. Cells were counted using a Bürker hemocytometer (total cells) and the bronchoalveolar lavage (BAL) fluid was centrifuged ( $1000 \times g$ , 10 min). For differential cell counts, 250  $\mu$ l of the resuspended cells (100,000 cells/ml) were spun ( $300 \times g$ , 6 min) (Cytospin 3, Shandon, TechGen, Zellik, Belgium) onto microscope slides, air-dried and stained (Diff-Quik® method, Medical Diagnostics, Düringen, Germany). For each sample, 200 cells were counted for the number of macrophages, eosinophils, neutrophils and lymphocytes.

After taking BAL fluid, the lungs were instilled with 4% formaldehyde until full inflation of all lobes, as judged visually. Evaluation of lung injury on slides stained by H&E was performed by an experienced pathologist who was blinded to the treatment given to the mice.

### 2.6. Total serum IgE

The OptEIA™ Mouse IgE set from Pharmingen (BD Biosciences, Erembodegem, Belgium) was used to measure total serum IgE (diluted 1/70). Measurements were performed according to the manufacturer's instructions.

### 2.7. Statistical analysis

All data were normally distributed as assessed by the D'Agostino & Pearson omnibus normality test. All data are presented as means and SD and were analysed using an unpaired  $t$ -test (lymphocytes characterization), a Kruskal–Wallis test followed by a Dunns multiple comparison test (airway hyperreactivity (AHR) and airway inflammation) or one-way ANOVA followed by a Bonferroni test (total serum IgE) (Graphpad Prism 4.01, Graphpad Software Inc, San Diego, USA). A level of  $p < 0.05$  (two tailed) was considered significant.

## 3. Results

### 3.1. Characterizing the cell suspension

Fig. 1 shows the subpopulations of cells obtained from auricular lymph nodes after two dermal sensitizations (day 15). Significantly higher amounts of antigen presenting cells (MHCII<sup>+</sup>) as well as the

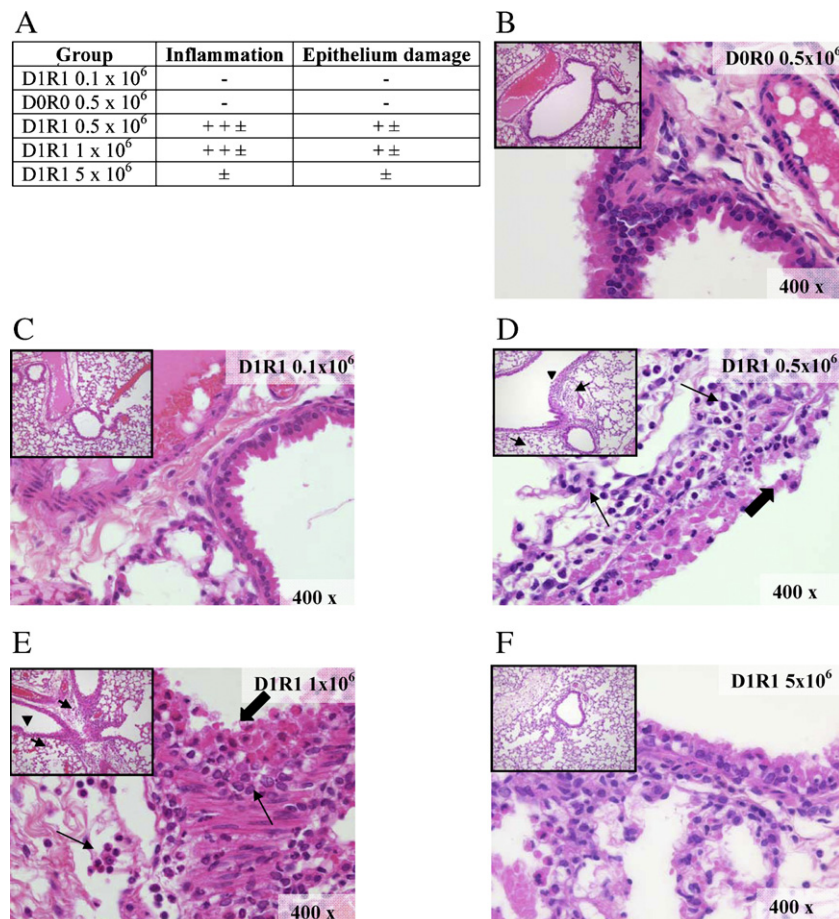


**Fig. 3.** Histology of lung tissue after transferring different amount of cells. H&E staining was performed on lung slices 24 h after challenging with the vehicle or TDI. The table (A) represents semi-quantitative scores on airway inflammation and epithelium damage. Data are presented with the following symbols: –, ±, +, ++, +++ – meaning no inflammation or epithelial damage and +++ meaning severe inflammation and epithelial damage. Figure (B–F) represent a large airway of the different treatment groups challenged with vehicle or TDI; a 400 $\times$  magnification with an insert of a 100 $\times$  magnification is shown of each treatment group. Symbols: (↑) inflammation (mainly neutrophils), (▲) epithelial shedding and (↑) epithelial necrosis. Experimental groups are identical to Fig. 2.

various lymphocyte subpopulations ( $CD3^+$ ,  $CD4^+$ ,  $CD4^+CD25^+$ ,  $CD8^+$  and  $CD19^+$ ) were found after TDI sensitization compared to controls. Total serum IgE was increased 4-fold in D1 ( $1537 \pm 702$  ng/ml) compared to D0 ( $396 \pm 245$  ng/ml).

### 3.2. Optimizing the amount of cells to be transferred

To find out the optimal quantity of cells to be transferred to naïve mice, we injected  $0.1 \times 10^6$ ,  $0.5 \times 10^6$ ,  $1 \times 10^6$  or  $5 \times 10^6$  cells of vehicle-treated or TDI-sensitized mice and three days later these mice were challenged with vehicle or TDI. Fig. 2A shows the AUC of R, measured with the FlexiVent system, after increasing concentrations of methacholine, 24 h after the challenge. A 2-, 3- and 2-fold increase in AHR was found after transferring  $0.5 \times 10^6$ ,  $1 \times 10^6$  or  $5 \times 10^6$  cells, respectively compared to their corresponding control groups.

Fig. 2B shows a higher amount of macrophages, neutrophils and eosinophils in the BAL of the D1R1  $0.5 \times 10^6$  group, compared to the D0R0  $0.5 \times 10^6$  and a higher amount of neutrophils in the D1R1  $1 \times 10^6$  group. Histology of the lungs confirmed the influx of macrophages, neutrophils and eosinophils in the D1R1  $0.5 \times 10^6$  and D1R1  $1 \times 10^6$  groups as well as epithelial necrosis and epithelial shedding of the large airways after TDI challenge (Fig. 3). Groups D1R1  $0.1 \times 10^6$  and D1R1  $5 \times 10^6$  showed no signs of inflammation neither in the BAL nor by histology.

A significant increase in total serum IgE was found after transferring  $1 \times 10^6$  cells of TDI sensitized mice (Fig. 2C).

### 3.3. Optimizing the time point for challenge after the transfer

With the results found in the first part of the experiments, we decided to proceed with the second part with transferring  $0.5 \times 10^6$  cells to the naïve mice.

Fig. 4A shows a significant increase in AHR in the D1R1 group only when the challenge was given 3 days after transferring the lymph node cell suspension.

A significant infiltration of neutrophils and eosinophils in the BAL fluid when challenging 3 days after the transfer (Fig. 4B) confirms the results found in Fig. 2.

Fig. 4C shows no significant differences in total serum IgE levels between the different days of challenge after transferring  $0.5 \times 10^6$  cells.

Fig. 5 shows a histological view of the lungs centered on a large airway, 24 h after challenging with TDI. Control animals (D0R0 d3) showed no signs of inflammation. The lungs of TDI-challenged animals showed an influx of neutrophils, epithelial necrosis and epithelial shedding in the D1R1 d1 and D1R1 d3 group.

## 4. Discussion

We have demonstrated that transferring different amounts of lymph node cells from TDI sensitized mice leads to different outcomes. Transferring  $0.5 \times 10^6$  cells gave, overall, the best results with regard to airway reactivity and airway inflammation. Furthermore, we established that challenging 3 days after the cell transfer leads, 22 h later, to an increased reactivity to methacholine and airway inflammation characterized by neutrophils and eosinophils. Challenging the mice earlier or later after transferring lymph node cells of TDI sensitized mice resulted in a less clear response.

Occupational asthma may account for 25% or more of de novo adult asthma (Dykewicz, 2009). Occupational asthma from sensitizers typically presents with a latent period of exposure, followed by the onset of clinical disease. After sensitization, airway reactions develop with lower levels of exposure to the agent that were tolerated before sensitization (Dykewicz, 2009; Maestrelli et al., 2009). The primary prevention of occupational asthma has, so far,

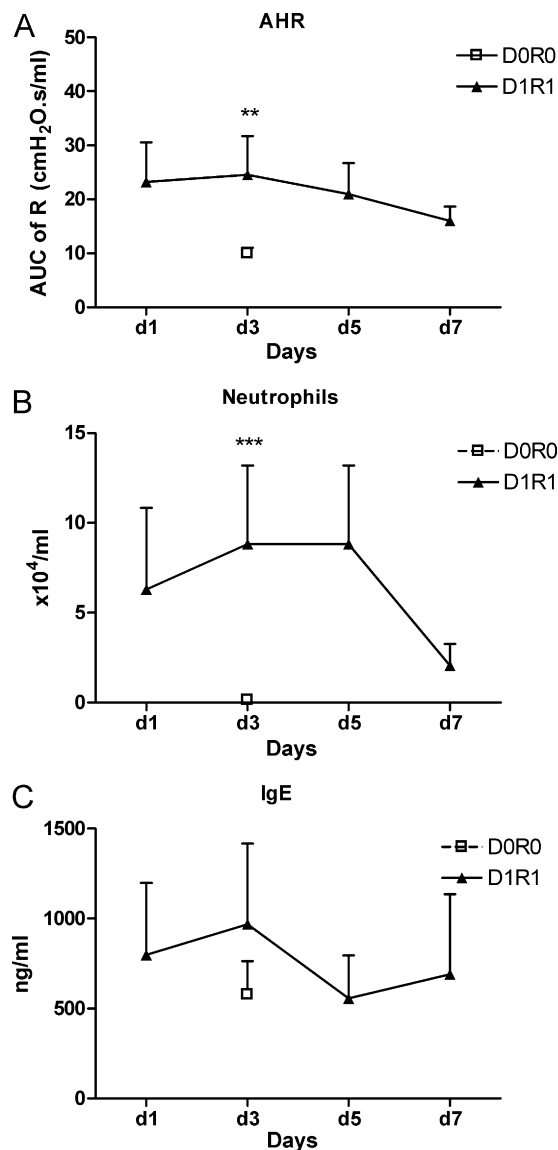


**Fig. 4.** Optimizing the time point for challenge after the transfer. The airway resistance (R), after increasing concentrations of methacholine (0–10 mg/ml), was measured 24 h after the challenge. The AUC of R was calculated (A). Total numbers of neutrophils were determined in the BAL fluid 24 h after challenging with the vehicle or TDI (B). Blood was collected 24 h after the challenge and total serum IgE was measured using a standard ELISA (C). Data are presented as mean  $\pm$  SD,  $n = 4$ –6 per group. \*\* $p < 0.01$  and \*\*\* $p < 0.001$  compared with the D0R0 d3 group. Experimental groups are D1R1 d1, D0R0 d3, D1R1 d3, D1R1 d5, D1R1 d7. D0R0 and D1R1 are identical to Fig. 2. Challenging the naïve mice was delayed until 7 days after the transfer of the auricular lymph node cell suspension (d1–7).

not been very successful. The annual reported number of cases has decreased only for isocyanates in the past decade (Karjalainen et al., 2001). Despite the slight decrease in incidence, asthma caused by isocyanates remains a health issue in workers.

Many aspects of the pathophysiology of occupational asthma are controversial. HMW agents and some LMW agents (acid anhydrides, platinum salts) induce asthma by producing specific IgE antibodies and these mechanisms are similar to allergic asthma that is unrelated to work (Maestrelli et al., 2009). The mechanisms by which other LMW agents (isocyanates, western red cedar) induce occupational asthma are less elucidated.

Using an established murine model of TDI-induced asthma we developed an adoptive transfer protocol (De Vooght et al., 2009; Tarkowski et al., 2007; Vanoirbeek et al., 2004, 2006, 2008,

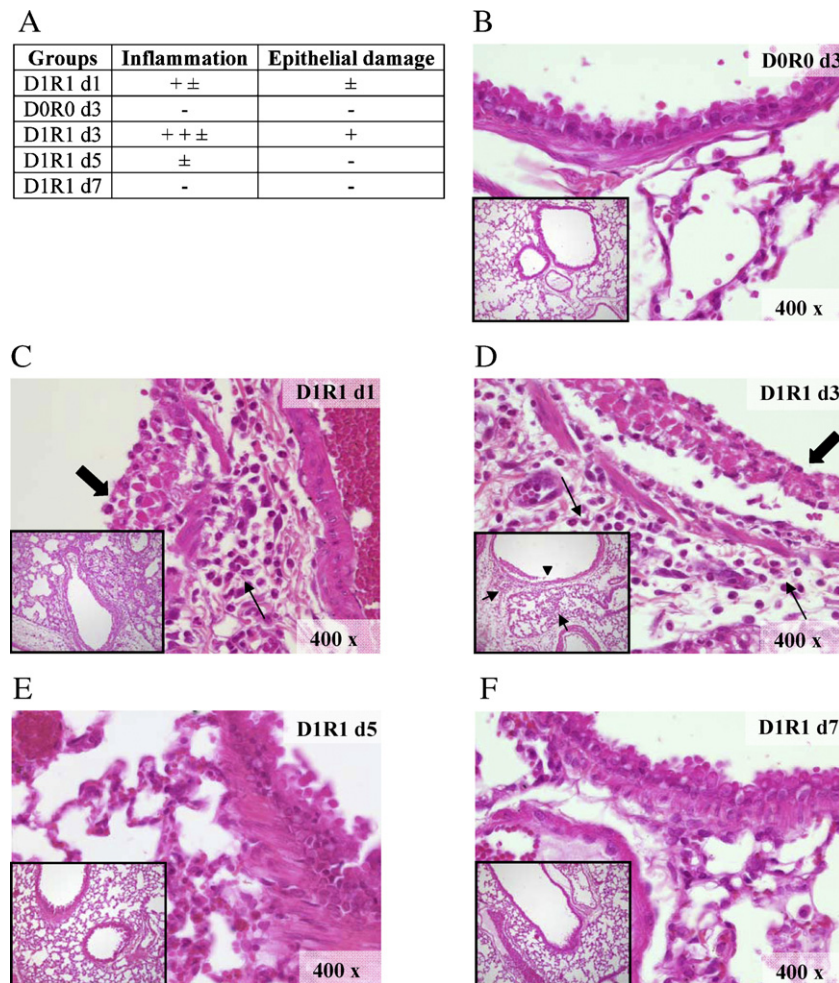


**Fig. 5.** Histology of lung tissue after challenging at different time points. H&E staining was performed on lung slices 24 h after challenging with the vehicle or TDI. The table (A) represents semi-quantitative scores on airway inflammation and epithelium damage. Symbols are identical to Fig. 2. Figures (B–F) are depicted as a 400× magnification with an insert of a 100× magnification. Symbols: (↑) inflammation (mainly neutrophils), (▲) epithelial shedding and (⬇) epithelial necrosis. Experimental groups are identical to Fig. 4.

2009). First we optimized the amount of cells to be transferred by administering intravenously  $0.1 \times 10^6$ ,  $0.5 \times 10^6$ ,  $1 \times 10^6$  or  $5 \times 10^6$  lymphoid cells. The increase in airway reactivity to methacholine seemed to be relatively independent of the amount of cells transferred, because  $0.5 \times 10^6$ ,  $1 \times 10^6$  and  $5 \times 10^6$  cells gave similar increases in AHR. The airway inflammation, however, differed substantially (although not significant) between transferring  $0.5 \times 10^6$ ,  $1 \times 10^6$  or  $5 \times 10^6$  cells. The transfer of  $0.5 \times 10^6$  cells gave an influx of neutrophils and eosinophils whereas  $1 \times 10^6$  cells gave a neutrophilic inflammation, while  $0.1 \times 10^6$  and  $5 \times 10^6$  cells gave no significant airway inflammation. At this moment we have no explanation for the apparent bell-shaped curves (Fig. 2). One possibility is that with transferring higher amounts of lymph node cells, more immune response competing cells are transferred resulting in a decrease of AHR and airway inflammation. Unique to our adoptive transfer protocol is the amount of cells we transfer. To our knowledge, there have been no articles published in which sensitization was transferred with such a low amount of cells. Scheerens et al. (1996) found an increase in AHR but no differences in airway inflammation after transferring, via the retro-orbital plexus,  $5 \times 10^6$  or  $1 \times 10^7$  lymphoid cells of the inguinal lymph nodes and spleen of TDI sensitized mice. Kearley et al. (2008) transferred  $0.5 \times 10^6$  CD4<sup>+</sup>CD25<sup>+</sup> cells to find a therapeutic effect in an ovalbumin mouse model, whereas Randolph et al. (1999) trans-

ferred  $5 \times 10^6$  or  $25 \times 10^6$  T-helper (Th) 1 and Th2 cells to find an increased airway inflammation. Cui et al. (2005) used adoptive transfer of  $3 \times 10^6$  in vitro-activated ovalbumin-specific Th2 cells into naïve mice, followed by ovalbumin challenge, and found an eosinophilic inflammation, hyperproduction of mucus and AHR. Although lymphocytes are the most abundant leukocyte type in the mice (approximately 1000/μl blood), we can state that transferring such large amounts of lymphocytes can cause leukocytosis and even signs of (pre)-leukemia (Nemzek et al., 2001; Opdenakker et al., 1998). Our goal was to transfer lower amounts of cells and remain within the physiological range of white blood cell counts. Therefore, although transferring  $1 \times 10^6$  cells resulted in a trend towards higher airway reactivity to methacholine, we prefer  $0.5 \times 10^6$  cells as an optimal amount for future experiments.

In a second part of the study we determined the optimal delay between transferring the complete lymphoid cell suspension and challenging the mice. Our protocol gave the best results in AHR and airway inflammation when the mice were challenged 3 days after transferring the lymphoid cells. Challenging one day after the transfer gave a non-significant trend to an increase in AHR and BAL neutrophils, whereas waiting longer to challenge the animals gave a decline (although not significant), especially in the airway inflammation. Lambert et al. (1998) transferred lymphoid cells from rats sensitized to house dust mite into wild type rats and prolonged the

time between transfer and challenge in recipient rats. They also found that challenging the rats three days after the transfer gave a maximum response.

In preliminary experiments two other control groups were included in the setup to be certain that our response was TDI specific. Transferring  $0.5 \times 10^6$  cells from AOO treated or TDI sensitized animals and challenging 3 days after the transfer with TDI (DOR1) or AOO (D1R0) respectively, gave no relevant increases in AHR or airway inflammation. These control groups were not used in our complete experiments to decrease the amount of animals needed.

To know the composition of the cell suspension we transferred, we analysed the suspension with flow cytometry. We checked for the presence of antigen presenting cells (MHCII<sup>+</sup>), Th lymphocytes (CD3<sup>+</sup>CD4<sup>+</sup>), T-cytotoxic lymphocytes (CD3<sup>+</sup>CD8<sup>+</sup>), T-regulatory/-activated lymphocytes (CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>) and B-lymphocytes (CD19<sup>+</sup>). After sensitization with TDI, this cell suspension consisted mostly of antigen presenting cells, Th and B lymphocytes. The total amount of the different cell types that were found will be used in the future to transfer the separate cell subsets to distinguish which cell types are important in chemical-induced asthma. We showed previously that in animals sensitized to TDI and challenged with TDI, both Th1 and Th2 cytokines are upregulated after TDI sensitization (data not shown) (De Vooght et al., 2009).

In conclusion we were able to passively transfer sensitization to TDI into naïve wild type animals using lymph node cells. This means that the cellular response alone can induce the typical phenotype of chemical-induced asthma.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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